Online Methods

Materials and plasmids.

Biotinylated peptides were synthesized at Stanford or Yale Protein and Nucleic Acid facilities as previously described[11]. Antibodies used in this study: anti-Histone H3 (Abcam), anti-Flag M5 (Sigma), anti-GST (Abcam), anti-tubulin (Upstate), anti-ORC2 (Upstate), anti-ORC3 (Abcam), anti-ORC5 (Sigma), anti-ORC6 (Abcam), anti-H4 (Abcam), anti-H4K20me1 (Abcam), anti-H4K20me2 (Abcam), anti-H4K20me3 (Abcam). ORC1 BAH domain was cloned into pGEX6P-1 for *in vitro* binding experiments, pBABE-puro-3XFLAG for generating cell lines stably expressing ORC1 proteins, pCDNA-HA-EGFP and pCAG-FLAG ORC1 for transfection in WI-38 cells, pcDNA for producing in vitro-transcribed mRNA for zebrafish microinjection. Site-directed mutagenesis was performed to introduce point mutations (Stratagene).

Cell culture, cell synchronization and transfection

293T, HT1080 and U2OS cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (ATCC), glutamine (Invitrogen) and penicillin/streptomycin (Invitrogen). U2OS cells were synchronized in 0.1ug/ml nocodazole (Sigma) for 24 hours. Cells were collected for ChIP analysis and flow cytometry 6 hours after releasing with DMEM. WI-38 human primary fibroblasts were maintained in DMEM/F12 (Invitrogen) with added supplements. Transient transfection was performed using TransIT-LT1 or TransIT-293 (Mirus) following the manufacturer's protocol.

Flow cytometry

Cells were collected and fixed in 70% ethanol at -20 ^{0}C for 15 mins and rehydrated in

PBS for 15 mins. Cells were then stained with 3 uM propidium iodide and 10 ug/ml Rnase A (Sigma) in staining buffer (100mM Tris, pH7.4, 150mM NaCl, 1mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% NP-40) for 30 mins.

Modified histone peptide microarrays

Peptide microarray experiments were performed as described previously[11]. Briefly, biotinylated histone peptides diluted in phosphate-buffered saline (PBS) supplied with 5% glycerol and 0.05% Tween-20 were printed on streptavidin-coated glass slides (ArrayIt) using VersArray Compact Microarrayer (BioRad). The arrays were probed with 25µg of GST-$ORC1_{BAH}$ and protein-peptide interactions were detected using anti-GST antibodies (Abcam) followed by Alexa Fluor 647 chicken anti-rabbit IgG antibodies (Invitrogen). To validate the epitope-specificity of H4K20me2 antibodies, the arrays were probed with 1µg of the indicated antibodies and the epitope-antibody bindings were detected by the same fluorophore-conjugated antibody as described above.

Biotinylated peptide binding assay

Biotinylated peptide pull-down assay was previously described[31]. Briefly, 1µg of peptide was incubated with 1µg of recombinant proteins in the binding buffer containing 50 mM Tris-pH 7.5, 150mM NaCl and 0.05% NP-40. Peptides were pulled down using streptavidin sepharose beads (Amersham) and protein-peptide bindings were detected by western analyses.

Full-length histone binding assay

Full-length histone pull-down assays were performed as previously described[32]. Briefly, 5µg of full-length histones purified from calf thymus (Wormington) was incubated with 25µg of recombinant proteins in the binding solution containing 50 mM Tris-pH 7.5, 150mM NaCl and 0.05% NP-40. Recombinant proteins were pulled down using glutathione sepharose 4B beads (Amersham) and bound histones were detected by Western analysis.

Protein expression and purification for structure analyses

For structure determination, the gene encoding residues 9-170 of mouse ORC1 was PCR amplified and inserted into a modified pRSFDuet-1 vector (Novagen), in which $mORC1_{BAH}$ was separated from the preceding His_6-SUMO tag by a ubiquitin-like protease (ULP1) cleavage site. The fusion protein was expressed in BL21 (DE3) RIL cell strain (Stratagene), in LB medium or minimum medium supplemented with 70 mg/l seleno-methionine for selenium labeling. The cells were grown at 37 °C and induced by 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when OD_{600} reached approx. 0.6. The temperature was then shifted to 20 °C for overnight culture growth. The fusion protein was purified through a Ni-NTA affinity column. The His_6-SUMO tag was removed by ULP1 cleavage, followed by a second round of Ni-NTA column purification, and gel filtration on a 16/60 G200 Superdex column. The final sample for crystallization of BAH_{mORC1} domain contains about 10 mg/ml $mORC1_{BAH}$ domain, 20 mM Tris-HCl, 50 mM NaCl, 5 mM DTT, pH 7.5.

For mutational analysis, mutants of the BAH domain human ORC1(1-185) were constructed by direct mutagenesis and purified as described above.

Crystallization conditions

For crystallization, the mORC1$_{BAH}$ domain was mixed with H4(14-25)K20me2 peptide in a molar ratio of 1:4. The crystallization condition (0.2 M sodium bromide, 25% PEG3,350, 20 °C) was initially identified using sparse-matrix screens (Hampton Research inc). The crystals were subsequently reproduced and improved by hanging-drop vapor-diffusion method, from drops mixed from 1 μl of H4K20me2-mORC1$_{BAH}$ domain solution and 1 μl of precipitant solution. This crystallization condition, when further supplemented with 0.2 M 3-(1-Pyridino)-1-propane sulfonate (Hampton Research inc), led to production of the crystals of free mORC1$_{BAH}$, even though the H4(14-25)K20me2 peptide was present in the crystallization solution. The crystals for both free and H4(14-25)K20me2-bound mORC1$_{BAH}$ domain were soaked in cryoprotectant made of mother liquor supplemented with 25% glycerol, before flash freezing in liquid nitrogen.

Structure determination

X-ray diffraction data sets for both free and H4(14-25)K20me2-bound mORC1$_{BAH}$ domain were collected at selenium peak wavelength on the 24-IDE NE-CAT beamline at the Advanced Photo Source (APS), Argonne National Laboratory. The diffraction data were indexed, integrated and scaled using the HKL 2000 program. The structure of free mORC1$_{BAH}$ was solved by the single-wavelength dispersion method with selenium atoms using the AutoSol program embedded in PHENIX software[33], which also gave an initial structural model. Further modeling of mORC1$_{BAH}$ domain was carried out using COOT[34], and was then subject to refinement using PHENIX. The final model was refined to 1.75 Å resolution.

The structure of H4K20me2(14-25)-bound mORC1$_{BAH}$ domain was solved by the molecular replacement method in PHASER[35] using the free structure of mORC1$_{BAH}$ domain as a

search model. The H4(14-25)K20me2 peptide was then modeled in COOT and the structure of the H4(14-25)K20me2-mORC1$_{BAH}$ domain was refined using PHENIX. The final model of the complex was refined to 1.95 Å resolution. For both free and H4(14-25)K20me2-mORC1$_{BAH}$ domain structures, the B-factors were refined with individual B values.

The statistics for data collection and structural refinement for both free and H4(14-25)K20me2-bound mORC1$_{BAH}$ domain are summarized in Supplementary Table 1.

ITC measurements

Protein and peptide samples used for ITC measurements were subject to overnight dialysis against buffer containing 20 mM Tris.HCl, 100 mM NaCl, 2 mM DTT, pH 7.5. Before the measurement, the protein and peptide concentrations were adjusted to about 0.1 mM and 1 mM, respectively. The ITC experiment was carried out using a MicroCal iTC200 instrument at 5 °C. The titration curves were analyzed using software Origin7.0 (MicroCal, iTC200).

Small-scale biochemical fractionation

Small-scale biochemical fractionation was modified from a protocol described previously[36]. In short, 1x10^7 – 2x10^7 were collected, washed with PBS, and resuspended in buffer A (10 mM HEPES [pH7.9], 10mM KCl, 1.5mM $MgCl_2$, 0.34M Sucrose, 10% Glycerol, 1mM DTT, Complete protease inhibitor tablet (Roche)). Trition X-100 was added to a final concentration of 0.1%. Cells were incubated for 8 minutes, and nuclei were collected by centrifugation (1,300 x g, 4^0C, 5 minutes). The supernatant (S1) was clarified by centrifugation at 20,000 x g, 4^0C for 5 minutes. The nuclei were washed once with buffer A and lysed in buffer B (3mM EDTA, 0.2 mM EGTA, 1mM DTT, Complete protease inhibitor tablet) for 30 minutes.

Chromatin was collected by centrifugation (1,700 x g, 4^0C, 5 minutes). The soluble fraction was combined with S1 and boiled at 90^0C with SDS sample buffer. Chromatin was washed with Buffer B once and resuspended in SDS sample buffer, boiled at 90 ^{0}C for 10 minutes.

Immunoprecipitation

HT1080 and U2OS cells stably expressing FLAG-ORC1 or FLAG-ORC1 mutants were generated by retroviral transduction. Cells were lysed in cell lysis buffer containing 50mM Tris-HCl (pH 7.4), 250 mM NaCl, 0.5% Triton X-100, 10% glycerol, 1mM DTT, Complete protease inhibitor tablet (Roche). Flag-ORC1 complexes was affinity purified by incubating anti-FLAG m2 mAb-conjugated agarose beads (Sigma) in lysates overnight at 4^0C. FLAG M2 beads were then washed 3 times with cell lysis buffer and bound protein eluted in SDS buffer for Western blot analysis.

ChIP analysis

Chromatin immunoprecipitation was performed as previously described[37]. The primer sequences used in this study:

MCM4 –5kbp FWD
TTC ACA TCC ACC CAG CTT ATC
MCM4 –5kbp REV
AGA GCA TTC TTC CCC TGA TG

MCM4 origin FWD
TTG GGT GGC TAC TTG GTG TT
MCM4 origin REV
TAG GCC CCT CGC TTG TTT

MCM4 +5kbp FWD
TTT TGA ATC TTG GTT TTG CTG A
MCM4 +5kbp REV
CAG CTT TTG GTT GGC TAA GG

β-globin –40kbp FWD
AGG TCA GGC CCT CAA GAG TC
β-globin –40kbp REV
CAG TCA GTT CTT TGG ACA AGT CTT A

β-globin origin FWD
TGA GCC TCT GCT GAT TCA TTT
β-globin origin REV
TTC AAG GGA GAG ACC TCA TTG T

β-globin +70kbp FWD
CTC AGA AGA ACC CTT GAT CTC C
β-globin +70kbp REV
TTC CGA GGA CAT TGG TTG A

Zebrafish aquaculture and microinjection

Wild-type AB zebrafish (*Danio rerio*) was maintained and raised using standard protocols. All experiments were conducted in accordance with AAALAC approved guidelines at Stanford University (protocol number: 10511). Embryos obtained from natural matings were microinjected at 1 to 2-cell stage with 1.5nL of 0.18mM sequence-specific *Orc1* morpholino or 0.36mM *Suv4-20h1/h2* combined morpholinos at 1:1 ratio targeting each gene's translation start site (GeneTools). For epistasis analyses, 1.5nL of morpholino solution containing 0.18mM *Orc1* morpholino and 0.36mM *Suv4-20h1/h2* combined morpholino (1:1 ratio) was used for microinjection. *orc1*: TCAGTCTTGTGATGTAGCGGCTCAT (as previously described[38]); *suv4-20h1*: ACCATGTTCTTGGATTCTCCCATCT; *suv4-20h2*: CACTCATTCTATAAGATCCCTCCAT. The morpholino targeting *orc1* splice site junction: ACAACTCTATTATGCTCACCTGTAC as previously described[38]. For the For reconstitution experiments, capped full-length ORC1 wild-type or H4K20me2-binding pocket mutant mRNAs were transcribed *in vitro* following the manufacturer's manual (Ambion). 10pg of mRNA was co-microinjected with *orc1* morpholino at the 1-cell stage. Embryos were cultured in E3 medium

at 28.5 ºC for 24 hours prior to scoring phenotypes. Live fish images were captured for individual fish using a Leica M205FA stereomicroscope and body length was calculated by Leica Application Suite program. Percentage of dwarf fish ($<AVG_{control}$-3X $STDEV_{control}$) in each experiment group was scored and p-values between groups were calculated by unpaired two-tailed Student's t test.

Reference

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